Performance of a whole blood interferon gamma assay in detecting latent infection with *Mycobacterium tuberculosis* in children

TG Connell MRCPI*1,2, N Curtis PhD*1,2, SC Ranganathan PhD2,3, JP Buttery FRACP1,2

* joint first authors

1 Infectious Diseases Unit, Department of General Medicine and Murdoch Children’s Research Institute, Royal Children’s Hospital Melbourne, Australia

2 Department of Pediatrics, University of Melbourne

3 Department of Thoracic Medicine, Royal Children’s Hospital Melbourne, Australia

**Corresponding Author**
A/Prof Nigel Curtis
Infectious Diseases Unit
Royal Children’s Hospital Melbourne
Flemington Road, Parkville 3052
Victoria, Australia
Tel: 61 (0) 3 9345 4545
Fax: 61 (0) 3 9345 6667
Email: nigel.curtis@rch.org.au

**Key words:** Tuberculosis, Interferon gamma, Tuberculin skin test, Mantoux, Diagnosis

The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non-exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd and its Licensees to permit this article to be published in Thorax editions and any other BMJPGL products to exploit all subsidiary rights, as set out in our licence (http://thorax.bmjournals.com/ifora/licence.pdf).
Abstract

Context
Diagnosis of latent *Mycobacterium tuberculosis* (MTB) infection using tuberculin skin testing (TST) in children is complicated by the potential influence of prior exposure to Bacille Calmette Geurin (BCG) vaccination or environmental mycobacteria. A whole blood assay has recently been developed to quantitatively measure interferon gamma (IFN-γ) production by lymphocytes specific to the MTB antigens ESAT-6 and CFP-10, but its use and assessment in children has been limited to date.

Objective
To compare the performance of the whole blood IFN-γ assay with TST in diagnosing latent tuberculosis (TB) infection or TB disease in children in routine clinical practice.

Patients
Children with a high risk of latent TB infection or TB disease were eligible. High risk was defined as contact with TB disease, clinical suspicion of TB disease, or recent arrival from an area of high TB prevalence.

Results
One hundred and six children were enrolled and of these, the whole blood IFN-γ assay was undertaken in 101. Seventeen (17%) of the 101 assays yielded inconclusive results due to failure of positive or negative control assays. There was poor correlation between the whole blood IFN-γ assay and the TST (kappa statistic 0.3) with 26 (70%) of the 37 children defined as latent TB infection by TST having a negative whole blood IFN-γ assay. There were no instances of a positive whole blood IFN-γ assay with a negative TST. Mitogen (positive) control IFN-γ responses were significantly correlated with age (Spearman’s coefficient = 0.53, p < 0.001), and in children with latent TB infection identified by TST, those with a positive IFN-γ assay were older (median 12.9 years vs. 6.92 respectively, p=0.007). The whole blood IFN-γ assay was positive in all nine children with TB disease.

Conclusion
There was poor agreement between the whole blood IFN-γ assay and TST for the diagnosis of latent TB. The whole blood IFN-γ assay may have lower sensitivity than the TST in diagnosing TB infection in children. A significant proportion of whole blood IFN-γ assays fail when used as a screening assay in routine practice.
Introduction

Globally, the burden of tuberculosis (TB) is immense with an estimated one third of the world’s population infected and projections that almost 12 million new cases will occur annually by 2006.[1] Accurate figures for the burden of childhood TB are not readily available but children represent an increasing proportion of the total number of cases.[2] In the majority of patients, primary infection with Mycobacterium tuberculosis (MTB) is contained by the host’s immune system (establishing latent infection). The cytokine interferon gamma (IFN-γ) produced by T cells has a critical role in the protective immunological response to primary infection.[3] [4] [5] Clinical latency can persist for many years with a lifetime risk of reactivation to active disease of approximately 10%.[3] [6] However, in children, up to 50% of untreated infants and 15% of older children with latent TB infection develop disease within two years of being infected.[7] Accurately identifying those who are latently infected is difficult as they remain asymptomatic. Currently, the tuberculin skin test (TST) is the established screening method for diagnosing latent TB infection in adults and children but has many potential problems. The specificity and sensitivity of this test is limited by prior Bacille Calmette Geurin (BCG) vaccination, previous exposure to environmental mycobacteria, and operator errors in placement and reading.[8] [9] [10] Recently, a whole blood assay (QuantiFERON – TB Gold, Cellestis LTD, Victoria, Australia) has been developed, and was approved by the US Food and Drug Administration in December 2004 as an *in vitro* test in adults for TB. The whole blood assay quantitatively measures IFN-γ production by previously sensitized lymphocytes in response to the MTB-specific proteins, early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10).[11] Potential advantages of the whole blood IFN-γ assay over TST include being unaffected by previous BCG vaccination, the higher specificity of the test (ESAT-6 and CFP-10 are absent from almost all environmental mycobacteria), the requirement for only one patient visit, and the exclusion of intradermal injection technique problems as well as elimination of errors in reading and interpretation. In addition to the whole blood assay, a rapid *ex vivo* enzyme linked immunospot assay (ELISPOT) has also been developed that counts individual antigen specific T cells. In this assay, each T cell that secretes IFN-γ in response to the TB-specific antigens ESAT-6 and CFP-10 leaves a footprint that can be enumerated using either an ELISPOT reader or a magnifying lens.[12] Studies in adults using the whole blood [13] [14] and the ELISPOT [15] [16] IFN-γ assays and, more recently, the ELISPOT assay in children [17] [18] have demonstrated that IFN-γ based assays have high sensitivity and specificity for TB disease, but none have focused specifically on the use of whole blood IFN-γ assays in the diagnosis of latent infection in children. The aim of this study was to compare the performance of the whole blood IFN-γ assay with TST in children referred for the evaluation of latent TB infection and TB disease.

Methods

Patients

Children (less than 18 years) with high risk of latent TB infection or TB disease referred to the Royal Children’s Hospital Melbourne, Australia between April 2004 and April 2005 were eligible for inclusion. High risk was defined as children with siblings or parents recently diagnosed with TB disease, clinical suspicion of TB disease, and those recently (within 5 years) immigrated from countries with a high prevalence of TB. Patients were recruited from the hospital wards (inpatients admitted for suspicion of clinical TB) and two hospital-based outpatient clinics, namely a TB clinic and a refugee health clinic. Demographic details...
recorded included age, country of birth, history of BCG vaccination (documented or verbally from parents) and the presence or absence of a typical BCG scar in the deltoid region, thigh or proximal forearm.

Tests
All patients underwent clinical evaluation, TST and whole blood IFN-\(\gamma\) assay. Chest X-ray was performed when clinically indicated or in those with a positive TST. TST was performed by intradermal instillation of 10 international units of tuberculin (Cellestis Tuberculin PPD 100 IU/mL, Melbourne, Australia) by trained staff, and read after 48 to 72 hours, measuring the transverse diameter of induration. Positive TST was defined as induration greater than 15 mm in individuals with evidence of prior BCG vaccination, greater than 5 mm in those who were known TB contacts (irrespective of BCG) and greater than 10 mm for all others.[7] The whole blood IFN-\(\gamma\) assay was performed in a certified clinical reference laboratory according to the manufacturer’s recommendations as described elsewhere.[9] In summary, aliquots (5 mL) of undiluted heparinized whole blood were incubated overnight with ESAT-6 and CFP-10, a negative control and the mitogen phytohaemagglutinin as a positive control. Following 16 to 24 hour incubation, plasma samples were harvested for IFN-\(\gamma\) quantification by a single-step sandwich-type ELISA. Concentration in whole blood of IFN-\(\gamma\) greater than 0.35 IU/mL as a response to exposure with either ESAT-6 or CFP-10 constituted a positive test.[13] The assay was deemed inconclusive if there was a high negative control (response with no antigen) or a negative mitogen control (indicating possible T cell anergy). In these instances the test was deemed to have ‘failed’ and was not repeated. The whole blood IFN-\(\gamma\) assay was performed as part of routine tests and the study was approved as a clinical audit by the hospital research ethics committee.

Definitions
In the absence of a recognized gold standard, and in agreement with published studies, latent TB infection was defined as an asymptomatic child with a positive TST as defined above and a normal chest X-ray. [16] [19] [20] TB disease was defined as a child with a positive TST who was symptomatic and/or had an abnormal chest x-ray consistent with TB and/or who clinically responded to anti tuberculous medication or a child with acid-fast bacilli detected, or MTB cultured or detected by molecular methods (PCR) from clinical specimens. Uninfected was defined as an asymptomatic child with a negative TST. The agreement between the whole blood IFN-\(\gamma\) assay and the TST for the diagnosis of latent TB infection was investigated.

Statistics
Data were analysed using Stata (Version 8.2, StataCorp, Texas). The median age of children in the latent TB group with a positive whole blood IFN-\(\gamma\) assay was compared with those with a negative assay using a Wilcoxon rank sum test. The measure of agreement between TST and whole blood IFN-\(\gamma\) assay for diagnosing latent TB infection was tested using the kappa statistic. The correlation between mitogen control response and age was assessed using the Spearman’s correlation coefficient.

Results
Clinical and demographic details are summarised in table 1. Of 106 children (61 males) enrolled in the study, five (5%) failed to return for TST reading and could therefore not be
assigned a diagnosis. Of the remaining 101, the majority (91%) originated from the Horn of Africa (Sudan, Somalia, Kenya and Ethiopia), reflecting recent migration demographics to Australia. On the basis of TST testing, 42 (41%) children were identified with latent TB infection; nine (9%) with TB disease and 50 (50%) were uninfected (Table 1). The median (range) age in the latent TB, TB disease and uninfected group was 9.2 (0.6-17.9) years, 3.9 (1.2-17.1) years and 6.8 (0.4-16.9) years respectively. Forty nine (49%) children had an identifiable BCG vaccination scar. Four (9%) children with a history of BCG vaccination had no scar. Only one child had written documentation of vaccination status including BCG.

Table 1 Demographic and clinical details

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Latent TB (n=42)</th>
<th>TB disease (n=9)</th>
<th>Uninfected (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median age years (range)</td>
<td>9.2 (0.6-17.9)</td>
<td>3.9 (1.2-17.1)</td>
<td>6.8 (0.4-16.9)</td>
</tr>
<tr>
<td>Male</td>
<td>23 (55%)</td>
<td>6 (67%)</td>
<td>32 (64%)</td>
</tr>
<tr>
<td>Born in high TB prevalence area</td>
<td>37 (88%)</td>
<td>7 (78%)</td>
<td>48 (96%)</td>
</tr>
<tr>
<td>TB contact</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Household†</td>
<td>24 (60%)</td>
<td>5 (56%)</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>Non household</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>7 (14%)</td>
<td>1 (11%)</td>
<td>8 (16%)</td>
</tr>
<tr>
<td>None</td>
<td>11 (26%)</td>
<td>3 (33%)</td>
<td>35 (70%)</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scar present</td>
<td>19 (45%)</td>
<td>3 (33%)</td>
<td>27 (54%)</td>
</tr>
<tr>
<td>History but no scar</td>
<td>2 (5%)</td>
<td>0 (0%)</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>No evidence of prior BCG</td>
<td>21 (50%)</td>
<td>6 (67%)</td>
<td>21 (42%)</td>
</tr>
<tr>
<td>Fevers</td>
<td>1 (2%)</td>
<td>7 (78%)</td>
<td>0</td>
</tr>
<tr>
<td>Night sweats</td>
<td>0 (0%)</td>
<td>6 (66%)</td>
<td>0</td>
</tr>
<tr>
<td>Cough &gt; 2 weeks</td>
<td>0 (0%)</td>
<td>6 (66%)</td>
<td>0</td>
</tr>
<tr>
<td>Tuberculin skin test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 5 mm</td>
<td>0 (0%)</td>
<td></td>
<td>37 (74%)</td>
</tr>
<tr>
<td>&gt; 5 - 10 mm</td>
<td>9 (22%)</td>
<td>2 (22%)**</td>
<td>9 (18%)</td>
</tr>
<tr>
<td>&gt; 10 - 15 mm</td>
<td>11 (26%)</td>
<td>1 (11%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>&gt; 15 mm</td>
<td>22 (52%)</td>
<td>3 (33%)</td>
<td>0</td>
</tr>
<tr>
<td>CXR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>38 (90%)</td>
<td>3 (33%)</td>
<td>12 (24%)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>0 (0%)</td>
<td>6 (66%)</td>
<td>0</td>
</tr>
<tr>
<td>Not done/Unavailable</td>
<td>4 (10%)</td>
<td></td>
<td>38 (76%)</td>
</tr>
</tbody>
</table>

** 3 children with TB disease did not have TST
† 34 with pulmonary TB; 1 with lymph node TB

The levels of response to CFP-10 and ESAT-6 from the whole blood IFN-γ assays are illustrated in figure 1. Of the 101 children, 98 (97%) had both TST and a whole blood IFN-γ assay performed (table 2). Seventeen (17%) of the whole blood IFN-γ assays ‘failed’ due to either a high negative control response (n=12) or an inadequate mitogen control response (n=5). Three patients diagnosed with TB disease did not have a TST performed due to concerns regarding potential ulceration.
There was poor correlation between the whole blood IFN-γ assay and the TST (kappa statistic 0.30) (table 2). Of 42 children with TST-defined latent TB infection, 37 had a successful whole blood IFN-γ assay and, of these, 26 (70%) were negative. Of these 37 children, 24 (65%) had a known TB household contact. There were no instances of a positive whole blood IFN-γ assay with a negative TST. The whole blood IFN-γ assay was positive in all 9 (100%) patients diagnosed with TB disease. TB disease was diagnosed in 3 children by PCR analysis of clinical samples. One further patient was diagnosed on the basis of clinical symptoms and granulomatous inflammation detected following biopsy of a para-vertebral mass. The remaining children were diagnosed based on a combination of suggestive clinical features, radiographic abnormalities and response to anti tuberculous treatment.

**Table 2** Results of whole blood IFN-γ assay by diagnostic group

<table>
<thead>
<tr>
<th>Diagnosis (based on TST)</th>
<th>Whole blood IFN-γ assay result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Uninfected</td>
<td>38 (76%)</td>
</tr>
<tr>
<td>Latent TB</td>
<td>26 (62%)</td>
</tr>
<tr>
<td>TB disease</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
</tr>
</tbody>
</table>

† 9 High negative control: 3 inadequate mitogen control  
* 3 High negative control: 2 inadequate mitogen control  
‡ 3 patients did not have TST

Of the 26 children with TST-defined latent TB and a negative whole blood IFN-γ assay, 13 (50%) had a TST induration greater than 15 mm and of all the children with TST induration greater than 15 mm, only 7/22 (31.8%) had a positive whole blood IFN-γ assay (figure 2).

Of the 42 children with TST-defined latent TB, twenty one (50%) had no evidence of prior BCG vaccination, and BCG status was similar in those with positive and negative whole blood IFN-γ assay results (11 of 26 negative assays compared with 7 of 11 positive assays had received BCG, ($\chi^2 = 0.68, p = 0.41$). Of the 21 BCG-unvaccinated children (no scar or history of immunization), 19 (90.5%) were from countries with a high prevalence of TB and the remaining 2 lived in families originally from a high prevalence countries. Nine of the 21 (42.8%) reported exposure to a TB household contact. In this subgroup of 21 children with a very high likelihood of latent TB infection, 16 (76.2%) whole blood IFN-γ assays yielded determinate results of which only 4 (25%) were positive.

In the whole blood IFN-γ assay, the quantitative mitogen ‘positive’ control response was significantly correlated with age (Spearman’s coefficient = 0.53, p < 0.001) (Figure 3). In those with TST diagnosed latent TB infection, a positive whole blood assay was also associated with increased age (median 12.9 years vs. 6.92 respectively, p=0.0065). In addition, children with positive whole blood IFN-γ assays tended to have higher mitogen control values (mean 27.73 IU/mL [95% CI 17.38, 38.08]) compared to those with negative assays (15.47 IU/mL [95% CI 12.71,18.23], p =0.03), probably reflecting that the former were older.
Discussion

Identifying and treating infectious cases is critical to TB control. In developed countries, where resources are more readily available and secondary prevention of reactivation disease feasible, considerable emphasis is also placed on identifying individuals latently infected with *M. tuberculosis*. [12] The burden of TB in developed countries is substantially borne by recent immigrants from high prevalence areas. [21, 22]

To our knowledge this is the first study investigating the performance of the whole blood IFN-γ assay in diagnosing latent TB infection in children. We have shown that in predominantly well children at high risk of latent TB infection, there is poor correlation between the TST and the whole blood IFN-γ assay, with 70% of children identified by TST to have latent TB infection having a negative whole blood IFN-γ assay result. This suggests that the whole blood IFN-γ has lower sensitivity than the TST or, less likely, that it is more specific revealing ‘false positive’ TST results. The former hypothesis is strongly supported by the finding that in 24 children deemed latently infected by TST and with documented household contact, the whole blood IFN-γ assay was positive in only 8 (33%) patients. Furthermore, out of a subgroup of 16 children with a very high likelihood of latent TB infection (all BCG unvaccinated, from families from high TB prevalence areas and TST induration ranging from 8 to 30 mm) only one quarter had a positive whole blood IFN-γ assay. Eight of these children had reported exposure to a TB household contact, of which only two had a positive whole blood IFN-γ assay.

Children with a positive whole blood IFN-γ assay were older and tended to have a higher mitogen control response. This association may have been due to cumulative probability of TB exposure or an increased ability to produce IFN-γ.

Of particular concern in our study was the relatively high ‘failure’ rate of the whole blood IFN-γ assay. Studies in adults using the whole blood IFN-γ assay, in a cohort of predominantly immunocompetent individuals, have reported indeterminate results in between 0.1 and 11% of assays. [23] However, a recent hospital-based study using a whole blood IFN-γ assay in routine clinical practice, reported indeterminate results in 21.4% of 318 patients. These were nearly all attributable to an inadequate mitogen control response. Indeterminate results were more frequent in patients with a negative TST and in patients receiving immunosuppressive medications. [24] In our study, 17% of the assays performed yielded inconclusive results, suggesting widespread application of the whole blood assay in children is questionable. The reason for these ‘failed’ tests is not apparent in our cohort of predominantly well children. In contrast to most previously published studies of whole blood IFN-γ assays for TB, we used routine testing facilities under normal clinical conditions, with no special provision for handling, transport or processing of samples. However, the state laboratory that performs the whole blood IFN-γ assays is a reference laboratory with over two years experience in the use of this assay, and performs in excess of 3000 assays per year. The 17% failure rate in our study therefore represents the ‘real world’ use of this assay in paediatric clinical practice.

High IFN-γ levels in the negative control have not been reported in children previously. Under most circumstances the negative control will not generate IFN-γ above the allowed threshold of 1.0 IU/mL. Results below this maximum threshold are subtracted from the result.
of the test specimen. A smaller number failed due to an inadequate mitogen control response. Decreased IFN-γ production to various stimuli has been well documented in infants, potentially increasing the chance of false negative assays, or requiring re-evaluation of lower threshold values for positive responses.[25] In agreement with published adult data, a threshold of 0.35 IU/mL IFN-γ was used to indicate a positive result. Using a lower cut-off of 0.1 IU/mL would yield only three more individuals with positive IFN-γ assays in the TB infected group. However, other studies have shown that IFN-γ production increases rapidly during infancy to reach adult levels.[26] [27] Of the 5 children with TST-defined latent TB infection and a negative mitogen control, none were infants. Furthermore, the youngest child in our study, who was 4 months of age, had a mitogen control response within the normal range.

Being unaffected by prior BCG vaccination, the new generation of IFN-γ assays hold much promise in reliably identifying patients requiring anti-tuberculous therapy. These *ex vivo* assays currently exist in two forms, an ELISA assay that directly measures IFN-γ production and an ELISPOT assay that counts individual antigen-specific T cells. These assays have been evaluated in adults in multiple settings described elsewhere.[28] In a study of South African children with TB *disease* with a high prevalence of HIV infection and malnutrition, Liebeschuetz *et al* demonstrated increased sensitivity of the ELISPOT assay compared to TST (83% vs 63%).[17] ELISPOT sensitivity was unaffected by age, HIV co-infection or malnutrition, all known to influence the TST. Combining both tests increased the sensitivity to 91%. Also, using an ELISPOT assay, Nicol *et al* reported a positive response in 83% of children with confirmed TB.[18] Of the 9 children diagnosed with TB in our study, all had positive whole blood IFN-γ assays.

The sensitivity of IFN-γ based assays among groups with a high likelihood of latent TB infection has also been reported.[19] [29] [30] In two school outbreaks, the ELISPOT assay was unaffected by BCG vaccination status, and showed significantly better correlation with MTB exposure than TST in 11-15 year old students [29], while the whole blood IFN-γ assay correlated well with TST results in Danish teenagers, and was also unaffected by BCG status.[30]. Recent studies in young infants and children using the ELISPOT assay have suggested superior sensitivity and specificity than the TST.[31, 32] In the absence of a gold standard test for latent TB, a number of investigators have chosen to report the agreement between TST and IFN-γ assays. Although variable, most studies in adults suggest an agreement between both whole blood INF-γ or ELISPOT assays and TST of between 60 and 80%.[28] A prospective comparison of the performance of the whole blood IFN-γ and ELISPOT assays in children with latent TB infection and disease would be valuable.

The effect of prior BCG vaccination on TST has been the subject of many reviews. A recent meta-analysis by Wang *et al* suggested that an induration of greater than 15 mm was more likely due to TB infection rather than prior BCG. [33] In our study BCG status did not appear to be associated with whole blood IFN-γ assay result in those with TST diagnosed latent TB infection. Those with positive and negative whole blood IFN-γ assay results were equally likely to have received BCG in the past.

Our study has highlighted the considerable disagreement between the TST and the whole blood IFN-γ assay in children at high risk for latent TB infection. Further studies are required to clarify the negative predictive value of this assay, particularly in children with possible latent TB infection[34].
Acknowledgements

We thank the participating staff and families involved in this study. This study received funding from the John Burge Trust, Victoria, Australia. Tom Connell is the recipient of a European Society of Paediatric Infectious Diseases (ESPID)/Wyeth fellowship award.

Competing Interest

None

Ethics

The study was approved by the Royal Children’s Hospital research ethics committee.

Figure legends

Figure 1
Results of whole blood IFN-γ assay for CFP-10 and ESAT-6. The dotted line depicts the test cut-off value for a positive result (0.35 IU/ mL).

Figure 2
The relationship between the whole blood IFN-γ assay results and TST induration diameter in the 42 patients with TST-defined latent TB infection. The bars depict the median value for each group.

Figure 3
Age related mitogen (positive) control response for different diagnostic categories.

References


